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ORIGINAL PAPER

Cindy L. Jellis · Sophia S. Wang · Paul Rennert
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Genomic organization of the gene coding for the costimulatory human B-lymphocyte antigen B7-2 (CD86)

Received: 8 December 1994 / Revised: 27 March 1995

Abstract The generation of an antigen-specific T-cell response requires that the T lymphocyte receive two signals from the antigen presenting cell. The specificity of this response is provided by antigen presented to the T lymphocyte and involves stimulation of the T lymphocyte via the T-cell receptor (TCR)/CD3 complex. The second, or costimulatory signal, can be provided by ligation of the B-lymphocyte activation antigens B7-1 (CD80) and B7.2 (CD86) to TCR antigen CD28. The cDNAs for both CD80 and CD86 have been isolated and are predicted to encode type 1 membrane proteins of the immunoglobulin (*Ig*) superfamily. The predicted protein is composed of a signal peptide followed by two *Ig*-like extracellular domains, a transmembrane domain, and a cytoplasmic tail. Here we report that the genomic organization of CD86 reflects its functional structure, and is similar to that found for CD80. The gene is composed of eight exons which span more than 22 kilobases. The predicted protein functional domains of signal peptide, extracellular *Ig*V- and *Ig*C-like regions, and transmembrane domain coincide with the genomic structure. Two independent sequences had been reported for CD86 cDNA which differed in their 5'untranslated (UT) regions. We find CD86 exons 1 and 2 correspond to these alternate 5'UT sequences. Splicing of exon 1 or 2 with the signal peptide encoding exon 3 would produce mRNA transcripts complementary to the reported cDNA clones. Exons 4 and 5

correspond to *Ig*V- and *Ig*C-like extracellular domains, respectively. Exon 6 encodes the transmembrane region and beginning of the cytoplasmic tail. Exons 7 and 8 encode the remainder of the cytoplasmic tail and 3'UT sequences.

Introduction

The induction of an immune response requires that the responding T cells receive both a primary signal delivered through the T-cell receptor (TCR)/CD3 complex (June 1991; Klausner and Samelson 1991) and a second or costimulatory signal (Jenkins and Schwartz 1987; Umetsu et al. 1987; Nisbet-Brown et al. 1987). The B-lymphocyte activation antigens B7-1 [(CD80) (Gimmi et al. 1991; Koulova et al. 1991; Linsley et al. 1991a, b; Freeman et al. 1991; Reiser et al. 1992) and B7-2 (CD86) (Boussiotis et al. 1993; Azuma et al. 1993; Freeman et al. 1993; Engel et al. 1994)] can deliver a costimulatory signal via the T cell CD28 pathway. Inhibition of the B7/CD28 binding in the presence of TCR signaling leads to the development of antigen-specific anergy (Gimmi et al. 1991). Blockage of the B7/CD28 pathway has led to the induction of antigen-specific tolerance and to engraftment of transplanted organs in a variety of animal models (Harding et al. 1992; Turka et al. 1992; Lenschow et al. 1992; Pescovitz et al. 1994; Blazar et al. 1994; Pearson et al. 1994).

The DNA sequence and predicted protein sequence of CD86 exhibits homology with CD80. Both proteins are composed of two extracellular domains related to the immunoglobulin (*Ig*) superfamily, a hydrophobic transmembrane region and a cytoplasmic domain. Despite their structural similarities and their ability to function as costimulatory molecules, CD80 and CD86 may have distinct roles. Both are expressed on activated B cells, activated monocytes, activated and resting dendritic cells, and activated T cells, and are absent from resting B and T cells (Freeman et al. 1993). CD86 alone appears on resting monocytes. While both CD80 and CD86 are expressed on

The nucleotide sequence data reported in this paper have been submitted to the EMBL/GenBank nucleotide sequence databases and have been assigned the accession numbers U17715 (HSB72S1), U17716 (HSB72S2), U17717 (HSB72S3), U17718 (HSB72S4), U17719 (HSB72S5), U17720 (HSB72S6), U17721 (HSB72S7), and U17722 (HSB72S8)

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B cells after induction, the kinetics and level of expression vary according to the inductive agent (Lenschow et al. 1994). Similarly, IFN- γ treatment of dendritic cells induces increased expression of CD86 alone (Larsen et al. 1994). The appearance of the two costimulatory molecules on different cell types at different times suggests that they may play unique roles in modulating an immune response.

Two CD86 cDNA sequences have been reported (Azuma et al. 1993; Freeman et al. 1993). These sequences are identical in their coding regions but diverge in their 5'UT and 3'UT sequences. In this study, we report the genomic structure of the human CD86 gene and show that both of the different reported 5'UT sequences exist as exons adjacent to the coding region.

Materials and methods

Screening the human genomic P1 library for CD86

The DuPont Merck Pharmaceutical Company Human Foreskin Fibroblast P1 Library #1 (DMPC-HFF#1) was screened for CD86 by Genome Systems (St. Louis, MO) using PCR primers complementary to CD86 cDNA (Freeman et al. 1993). The four DNA primers used for polymerase chain reaction (PCR) screening of this library were as follows:

"J5", 5'-CACGGTTACCCAGAACCTAAG-3'; "J6", 5'GGTGAAGATAAAAGCCGCGTC-3'; "J7", 5'-CACAGGGTGAAAGCTTTGC-3'; and "J8", 5'-GGGATCCATTTGGCTGC-3'.

PCR primers J5 and J6 are sense and antisense primers derived from the IgC-like domain of CD86 and generate a 212 base pairs (bp) PCR product. PCR primers J7 and J8 are sense and antisense primers derived from the 5'UT CD86 sequence published by Freeman and co-workers (1993) and generate a 115 bp PCR product. PCR conditions used to identify CD86-specific P1 clones consisted of 30 cycles each composed of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min.

Preparation of DNA from CD86 genomic P1 clones for direct sequencing

P1 DNA was transferred into *Escherichia coli* (*E. coli*) host DH10B to allow preparation of DNA suitable for direct double-strand cycle sequencing. This was accomplished by two rounds of Hfr-mediated mating utilizing *E. coli* strains NS3529 and DH10B and protocols provided by Genome Systems. DH10B cells containing DMPC-HFF#1 P1 clone 1390-A1 or clone 1307-7C DNA were selected based on their acquired resistance to chloramphenicol (encoded by the F' episome) and kanamycin (encoded on the P1 plasmid). Plasmid DNA suitable for direct sequencing was prepared from a 500 ml yeast tryptone broth culture containing kanamycin (25 μ g/ml) that had been inoculated with 5 ml of an overnight culture. The cultures were incubated at 37 °C at 250 RPM for 30 min, IPTG was added to a final concentration of 1 mM to induce the P1 lytic operon and cells were harvested 5 h post induction. The cells were collected by centrifugation and P1 plasmid DNA prepared by the triton lysis method (protocol provided by Genome Systems). Final yield of P1 DNA was approximately 25 μ g/500 ml culture.

Sequencing CD86 P1 clones

Oligonucleotide primers complementary to CD86 cDNA were used to sequence exon/intron boundaries in both directions. Cycle sequencing was carried out with an ABI (Foster City, CA) prism kit using 1 μ g of P1 template DNA, 4.8 pmol primer, and 30 cycles each consisting of

denaturation at 97 °C for 15 s, followed by annealing at 55 °C for 15 s, and extension at 60 °C for 4 min. Reactions were purified on "Bakerbond spe" (J. T. Baker, Phillipsburg, NJ) 1-ml disposable filtration columns and the DNA was precipitated with the addition of 2.5 volumes of ethanol. Precipitated DNA was resuspended in 5 μ l of a 4:1 mixture of formamide: 50 mM ethylenediaminetetraacetate, heated for two min at 95 °C, placed on ice, and the sequence determined using an ABI 373A automated sequencer (ABI).

Southern blot analysis of restriction enzyme-digested P1 DNA

For Southern blot analysis (Southern 1975), DNA was prepared from 500 ml IPTG-induced cultures of CD86 genomic P1 clones #1390-A1/DH10B and #1307-7C/DH10B using QIAGEN (Chatsworth, CA) protocols and reagents. Approximately 1 μ g of plasmid DNA per reaction was digested with a panel of restriction endonucleases, and the digested DNA analyzed by agarose gel electrophoresis. The DNA was visualized by staining with ethidium bromide and transferred to nitrocellulose membrane filters. Oligonucleotides corresponding to CD86 exons and flanking intron sequences were synthesized and 3' end-labeled with fluorescein-dUTP (ECL 3' oligolabeling and detection system; Amersham, Arlington Heights, IL). Southern blots of restriction enzyme-digested DNA prepared from both clones were probed with fluorescein dUTP-labeled oligonucleotides. The hybridized oligonucleotides were detecting using anti-fluorescein HRP conjugate antibodies (Amersham). The sizes of the restriction fragments were estimated by comparison with ECL DNA markers (Amersham) designed for use with this system.

Confirmation of intron sizes by PCR

The length of introns determined by Southern blot analysis were confirmed by PCR analysis using primers complementary to exon sequences at the exon intron boundaries and directed towards adjacent exons. Template DNA (20 ng) and 100 pmol of each sense and antisense primer were mixed and denatured at 97 °C for 2 min. The intron sequences were PCR amplified using 30 cycles each composed of a denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 4 min. PCR products were analyzed on agarose gels, transferred to nitrocellulose, and probed with a variety of labeled intron and exon primers to measure size and to confirm integrity.

Results

Two human genomic P1 clones were identified by probing with CD86-specific PCR primers provided by Genome Systems. P1 human genomic clone 1390-A1 was positive in PCR reactions with primers J7 and J8, specific for CD86 5'UT sequences, and negative in PCR reactions using the IgC-like domain primers J5 and J6. P1 human genomic clone 1307-7C gave the expected 212 bp PCR product with IgC-like domain primers J5 and J6 and was negative in PCR reactions, using the 5'UT primers J7 and J8. Southern blots of restriction enzyme-digested clone 1390-A1 and clone 1307-7C P1 DNA were probed with exon-specific-labeled oligonucleotides to assign CD86 exons to their respective genomic clones. Both reported 5'UT exon sequences were localized to P1 clone 1390-A1. The signal peptide, Ig-like extracellular, transmembrane, and cytoplasmic domains were localized to P1 clone 1307-7C.

DNA sequencing of intron/exon boundaries was performed directly on P1 CD86 genomic DNA using primers derived from CD86 cDNA sequences. All junctions were

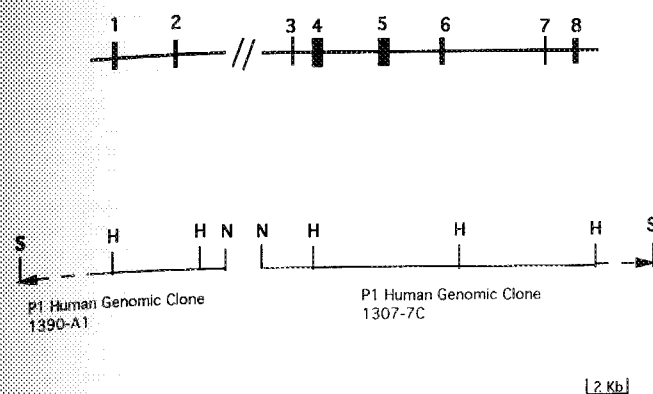


Fig. 1 Exon-intron organization of the human *CD86* gene. The *CD86* gene contains eight exons shown as solid boxes. Exons 1 and 2 are alternatively spliced to exon 3 to yield different *CD86* mRNA transcripts. P1 clones containing *CD86* genomic sequences are shown below, along with recognition sites for *Hin* dIII (H), *Not* I (N), and *Sfi* (S) restriction endonucleases. The broken line indicates genomic sequences present in P1 clones which extend for an additional 60–70 kb as estimated from the packaging requirements for bacteriophage P1 (Sternberg 1990) and measured here

found to have conventional splice signal sequences (Shapiro and Senapathy 1987) and the *CD86* coding sequences were found dispersed over eight exons. Exons 1 and 2 encode the 5'UT sequences reported by Freeman and co-workers (1993) and by Azuma and co-workers (1993), respectively. Exon 3 encodes the predicted signal peptide and exons 4 and 5 encode the two Ig-like extracellular domains. Exon 6 encodes the transmembrane and part of the cytoplasmic domain. Exons 7 and 8 encode a portion of the cytoplasmic domain and the remainder of the cytoplasmic and 3'UT domains, respectively.

Introns were sized by Southern blot analysis of restriction enzyme-digested DNA and by PCR using primers directed across the introns from bordering exons. The results show that the *CD86* gene spans at least 23 kilobases [(kb) (Fig. 1)]. The length of the introns determined ranged in size from 1.2 kb to 5.5 kb as shown in Figure 2. The length of intron B separating exons 2 and 3 was greater than 4.3 kb. The exact length was not determined because *CD86* genomic clones 1390-A1 and 1307-7C do not overlap. This was confirmed by probing Southern blots of clone 1390-A1 restriction enzyme-digested DNA with a fluorescein dUTP-labeled oligonucleotide corresponding to the N-terminal sequences of *CD86* genomic clone 1307-7C. The N-terminal sequences of clone 1307-7C were determined by direct sequencing using T7 primer sequences located in the vector adjacent to the cloned genomic DNA. These T7 primer sequences are adjacent to the *Not* I restriction site at the N-terminus of 1307-7C shown in Figure 1. The nucleotide sequences of the *CD86* exons along with several hundred bases of bordering intron sequences are shown in Figure 2.

Discussion

The two independent cDNA sequences reported for the *CD86* gene contained identical coding sequences for the *CD86* protein region but had different 5'UT sequences. The existence of both of the 5'UT sequences was confirmed by DNA sequencing of the *CD86* genomic DNA. In the *CD86* gene, exons 1 and 2 correspond to these alternate 5'UT regions. The isolation of the two *CD86* cDNAs suggests that the expression of this gene may be regulated at the transcriptional or RNA splicing level, which could give rise to tissue-specific expression of *CD86*. The *CD86* cDNA isolated by Freeman and co-workers (1993) was found in a library prepared from activated human B lymphocytes, while that reported by Azuma and co-workers (1993) was found in a library prepared from the JY B-lymphoblastoid (B-LBL) cell line. The alternate 5'UT regions found in the two cDNAs may reflect this difference in the source of cell for the preparation of the respective libraries. The JY B-LBL cell line constitutively expressed *CD86* at high levels and was the source of the *CD86* cDNA which started with exon 2. Activated human B lymphocytes were the source of the cDNA starting with the exon 1 sequence. The two *CD86* cDNA with alternate 5'UT sequences may represent constitutively expressed and inducible forms of *CD86*. We found the divergence of these *CD86* cDNA sequences to occur precisely at exon 3 (Fig. 2), which encodes the predicted secretory signal peptide. An exon 1/3 splice event gives rise to the cDNA clone isolated from activated human B lymphocytes as described by Freeman and co-workers (1993). Alternatively, splicing of exon 2 to exon 3 produces the *CD86* cDNA clone isolated from the B-LBL cell line reported by Azuma and co-workers (1993). In both cDNAs, translation of the signal peptide probably initiates at the methionine codon in exon 3. The organization of the *CD86* genome reflects the structural aspects of the *CD86* protein. The signal peptide sequence, the two Ig family member Ig-like extracellular domains, and the transmembrane domain are located on individual exons. The cytoplasmic domain of *CD86* is split between three exons (exons 6, 7, and 8) suggesting the possibility that alternate splicing could give rise to *CD86* with different intracellular sequences. While alternate splicing of the cytoplasmic domain of *CD86* has not been reported, multiple cytoplasmic domains do exist for the mouse homologue of *CD80* (Borriello et al. 1994).

The genomic organization of *CD86* is similar to that reported for *CD80*. The 5'UT sequences were found on a separate exon. In *CD86*, exon 3 encodes a signal peptide, exon 4 contains the predicted IgV-like domain, exon 5 contains an Ig C-like domain, and exon 6 encodes a stretch of hydrophobic residues corresponding to the transmembrane domain. These regions correspond to exons 2 through 5 in *CD80*, respectively (Selvakumar et al. 1992). The genomic organization of *CD80* and *CD86* diverges at the 3' end of the gene. Notably, the cytoplasmic tail for *CD86* spans three exons (exons 6 through 8) in contrast to being entirely contained in the final exon for *CD80*. No evidence

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